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(71) Applicant *(for all designated States except US)*: PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 800 Capital Square, 400 Locust Street, Des Moines, IA 50319 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BASZCZYNSKI, Christopher, L. [CA/US]; 7305 Benton Drive, Urbandale, IA 50322 (US). LYZNIK, Leszek, Alexander [PL/US]; 5960 Greendale Place #105, Johnston, IA 50131 (US). GORDON-KAMM, William J. [US/US]; 3916 67th Street, Urbandale, IA 50322 (US). RAO, Aragula, Gururaj [US/US]; 4734 74th Street, Urbandale, IA 50322 (US). TAGLIANI, Laura, A. [US/US]; 7552 N.W. 28th Street, Ankeny, IA 50021 (US). GUAN, Xueni [CN/US]; 3668 Ruette de Ville, San Diego, CA 82139 (US).

(74) Agents: SPRUILL, W., Murray et al.; Bell Seltzer Intellectual Property Law Group, Alston & Bird LLP, P.O. Drawer 34009, Charlotte, NC 28234 (US).

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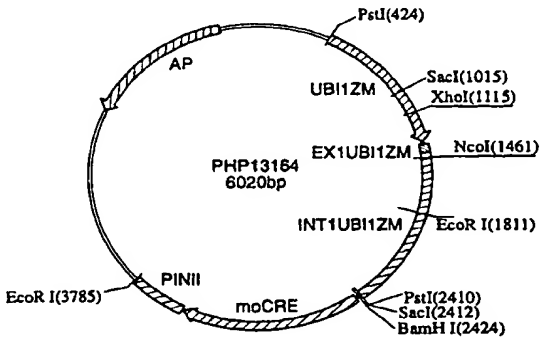
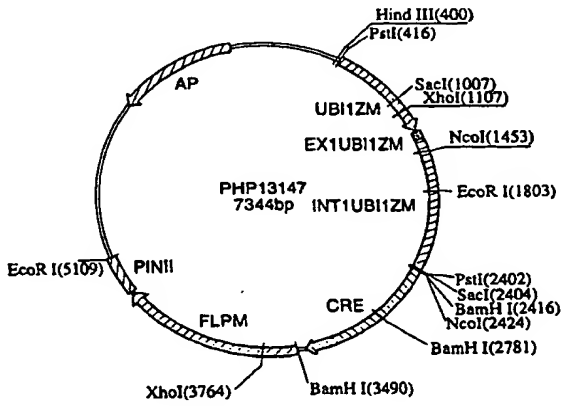
(54) Title: A NOVEL METHOD FOR THE INTEGRATION OF FOREIGN DNA INTO EUKARYOTICGENOMES

(57) Abstract

Compositions and methods for introducing a DNA of interest into a genomic target site are provided. In particular, the methods and compositions involve the use of a combination of target sites for two site specific recombinases and expression of a chimeric recobminase with dual target site specificity. Thus, the compositions comprise novel site-specific recombinases with specificities to multiple target sites, and nucleotide sequences and expression cassettes encoding these recombinases or target sites. The methods involve transforming a eukaryotic cell having target sites for the novel recombinase with a DNA of interest that is flanked by corresponding target sites. Expression of the recombinase results in integration of the DNA of interest into the genome of the cell. The compositions and methods of the invention have use in the construction of stably transformed eukaryotic cells, and in particular, plant cells. The methods result in the efficient targeted genomic intergration of DNA by site-specific recombination.





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A NOVEL METHOD FOR THE INTEGRATION OF FOREIGN DNA INTO EUKARYOTIC GENOMES

FIELD OF THE INVENTION

The invention relates to the genetic modification of chromosomes. In particular, methods and compositions for the integration of DNA into a eukaryotic genome are provided.

BACKGROUND OF THE INVENTION

Several approaches have been used to integrate a DNA of interest into the genome of a plant. In the simplest method, DNA is introduced into a cell and randomly integrates into the genome through illegitimate recombination. One drawback to this method is that positional effects due to random integration make gene expression difficult to analyze.

As an alternative to illegitimate recombination, integration may be targeted to a particular site on the genome through the use of homologous recombination or site-specific recombination. In plants, where homologous recombination technology has not been developed, site-specific recombination is used to integrate a sequence of interest into an integration site that has been previously inserted into the plant host genome. If site-specific integration occurs by a single cross-over event between a chromosome and a circular extrachromosomal replicon, the entire replicon will be inserted into the chromosome. When insertion of the entire replicon is undesirable, a fragment of the replicon comprising the DNA of interest, flanked by target sites for a site-specific recombinase, may be introduced by a double reciprocal cross-over event, into a chromosome having an integration site corresponding to the target sites which flank the DNA of interest. In either case, integration is inefficient because it is reversible, that is, the integrated DNA may be excised by subsequent site-specific recombination between the target sites flanking the integrated DNA.

Several approaches have been taken to avoid excision of an integrated DNA. In one approach, expression of a site-specific recombinase, such as Cre or FLP, is temporally regulated. See O'Gorman *et al.* (1991) *Science* 251:1351-1355; Logie and Stewart (1995) *Proc Natl Acad Sci* 92:5940-5944; Zhang *et al.* (1996) *Nuc Acid Res* 24:543-548; Nichols *et al.* (1997) *Mol Endocinol* 11:950-961; and Feil *et al.* (1997) *Biochem Biophy Res Comm* 237:752-757; the contents of which are incorporated by reference. In these methods, the recombinase is briefly expressed, either transiently or inducibly, in order to allow integration. However, excision of the integrated DNA may occur before active recombinase disappears from the cell. Furthermore, intramolecular excision is kinetically favored over bi-molecular integration. Therefore, integrated DNA is inherently unstable in the presence of recombinase.

A second approach reduces excision of integrated DNA by using pairs of singly mutated target sites on both the chromosome and flanking the DNA of interest. See Albert *et al.* (1995) *Plant J* 7:649-659; Schlake and Bode (1994) *Biochemistry* 33:12746-12751; O'Gorman *et al.* (1997) *Proc Natl Acad Sci* 94:14602-14607; and Araki *et al.* (1997) *Nuc Acid Res* 25:868-872; the contents of which are incorporated herein by reference. Recombination between singly mutated target sites results in doubly mutated target sites flanking the DNA inserted into the chromosome. The doubly mutated target sites are not well recognized by the recombinase. Thus, the inserted DNA is excised from the chromosome by a reverse reaction only at low levels. This system, however, has the disadvantage that the singly mutated target sites often do not act as efficient recombination substrates and thus the frequency of integration is reduced. In addition, transformants are unstable because excision may still occur, although at reduced frequency.

Accordingly, it is an object of the invention to provide efficient methods for site-specific integration of DNA into eukaryotic genomes which avoid subsequent excision reactions and other non-productive recombination reactions.

SUMMARY OF THE INVENTION

Compositions and methods for introducing a DNA of interest into a genomic integration site are provided. In particular, the methods and compositions involve the use of a combination of target sites for two distinctive site-specific recombinases, such as Cre and FLP, and expression of a chimeric recombinase with dual target site specificity. Thus, the compositions comprise novel site-specific recombinases with specificities to multiple target sites, and nucleotides sequences and expression cassettes encoding these recombinases or target sites. The methods involve transforming a eukaryotic cell having target sites for the novel recombinase with a DNA of interest that is flanked by corresponding target sites. Expression of either the novel chimeric recombinase or two site-specific recombinases in the eukaryotic cell results in integration of the DNA of interest into the genome. The compositions and methods of the invention have use in the construction of stably transformed eukaryotic cells, and in particular, plant cells. The methods result in the efficient targeted genomic integration of DNA by site-specific recombination.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically represents plant transformation vectors, PHP13164 and PHP13147, for expression of moCRE recombinase and Cre:FLPm recombinase, respectively.

Figure 2 graphically represents activation of GUS expression by FLPm or CRE:FLPm mediated excision of a sequence flanked by FRT sites that separates the ubiquitin promoter and the GUS open reading frame.

Figure 3 graphically represents activation of GUS expression by CRE:FLPm mediated excision of a sequence flanked by loxP sites that separates the ubiquitin promoter and the GUS open reading frame.

DETAILED DESCRIPTION OF THE INVENTION

Compositions and methods for site-specific integration of DNA into predetermined genomic integration sites in a host genome are provided. The invention provides for the use of chimeric recombinases that catalyze site-specific recombination between target sites that originate from different site-specific recombination systems. Such a dual function chimeric recombinase ensures that the two ends of foreign DNA do not ligate with each other, but instead, recombine with their cognate partner target sites residing in the genomic DNA. The methods facilitate the directional targeting of desired genes and nucleotide sequences into corresponding integration sites previously introduced into the genome.

In the methods of the invention, a combination of target sites for two site-specific recombinases are introduced into the genome of an organism of interest, establishing an integration site for insertion of nucleotide sequences of interest. For the purposes of the invention, an integration site will comprise flanking target sites where the target sites correspond to the recombination sites for two distinctive site-specific recombinases. These recombination or target sites may flank other nucleotide sequences or may be contiguous. Methods for the production of transgenic plants containing specific recombination sites integrated in the plant genome are described in co-pending provisional application, serial No. 60/065,627, entitled ACompositions and Methods for Genetic Modification of Plants,@ filed 18 November 1997, and herein incorporated by reference. Once a stable plant or cultured tissue is established, a transfer cassette comprising a DNA of interest, flanked by target sites corresponding to those of the genomic integration site, is introduced into the stably transformed plant or tissues in the presence of a chimeric recombinase with specificities to each of the target sites. Alternatively, two distinct recombinases corresponding to the target sites may be present in the cell in lieu of a chimeric recombinase. This process results in exchange of the nucleotide sequences between the two identical target sites of the genomic integration site and the transfer cassette.

Thus, the invention provides a method for integrating a DNA of interest into the genome of a eukaryotic cell, comprising:

a) transforming said cell with a transfer cassette comprising said DNA, wherein said DNA is flanked by a target site for a first site-specific recombinase and a target site for a second site-specific recombinase, and said genome contains an integration site comprising target sites corresponding to said target sites flanking said DNA; and

b) providing in said cell a recombinant protein comprising said first recombinase fused in frame with said second recombinase.

The invention further provides a method for integrating a DNA of interest into the genome of a eukaryotic cell, comprising:

a) transforming said cell with a transfer cassette comprising said DNA, wherein said DNA is flanked by a target site for a first site-specific recombinase and a target site for a second site-specific recombinase, and said genome contains an integration site comprising target sites corresponding to said target sites flanking said DNA; and

b) providing in said cell said first recombinase and said second recombinase.

By "site-specific recombinase" is meant any enzyme that catalyzes conservative site-specific recombination between its corresponding recombination sites. For reviews of site-specific recombinases, see Sauer (1994) *Current Opinion in Biotechnology* 5:521-527; and Sadowski (1993) *FASEB* 7:760-767; the contents of which are incorporated herein by reference.

The first and second site-specific recombinases may be full length recombinases and/or active fragments or derivatives thereof. Site-specific recombinases useful for creating the chimeric recombinases of the invention, include recombinases from the integrase family, derivatives thereof, and any other naturally occurring or recombinantly produced enzyme or derivative thereof, that catalyzes conservative site-specific recombination between specified DNA sites. The

integrase family of recombinases has over thirty members and includes FLP, Cre, Int and R. Preferably, the recombinases do not require cofactors or a supercoiled substrate. Most preferably the recombinases are Cre and FLP. The bacteriophage P1 *loxP*-Cre and the Saccharomyces 2μ plasmid FRT/FLP site-specific recombinations systems have been extensively studied and their uses are well known to those skilled in the art. Cre and FLP are known to function in a variety of organisms, including bacteria, yeast, Drosophila, mammals and monocotyledonous and dicotyledonous plants. In addition these recombinases do not require auxiliary factors to function.

The site-specific recombinases and sequences encoding them that are used in the methods and compositions of the invention may be variants of naturally occurring recombinases and the genes encoding them. The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or conservatively modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations" and represent one species of conservatively modified variation. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid

with a chemically similar amino acid. Thus, any number of amino acid residues selected from the group of integers consisting of from 1 to 15 can be so altered. Thus, for example, 1, 2, 3, 4, 5, 7, or 10 alterations can be made. Conservatively modified variants typically provide similar biological activity as the unmodified polypeptide sequence from which they are derived. For example, substrate specificity, enzyme activity, or ligand/receptor binding is generally at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the native protein for it=s native substrate. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

See Creighton (1984) Proteins, W.H. Freeman and Company.

Rather than use full length recombinases, functional fragments of site-specific recombinases may be used in the methods and compositions of the invention. Functional fragments of site-specific recombinases can be identified using a variety of techniques. For example, functional fragments of the FLP protein may be identified by their ability, upon introduction to cells containing appropriate FRT substrates, to catalyze site-specific recombination and result in the excision of an assayable marker gene.

A general approach of such functional analysis involves subcloning DNA fragments of a genomic clone, cDNA clone or synthesized gene sequence into an expression vector, introducing the expression vector into a heterologous host, and screening to detect the product of recombination (*i.e.* using restriction analysis

to verify the product of recombination at the nucleic acid level, or relying on an assay system for recombination as described above). Methods for generating fragments of a cDNA or genomic clone are well known. Variants of an isolated DNA encoding a site-specific recombinase can be produced by deleting, adding and/or substituting nucleotides. Such variants can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like. See, for example, Ausubel, Current Protocols In Molecular Biology, Wiley Interscience (1990) pages 8.0.3 - 8.5.9, and McPherson (ed.), *Directed Mutagenesis: A Practical Approach*, (IRL Press, 1991).

The dual function recombinant proteins of the invention comprise a first site-specific recombinase fused in frame with a second site-specific recombinase. It will be recognized that in the methods of invention, the recombinases comprising the chimeric recombinase must correspond to the target sites of the transformed organism and the targeting cassette. That is, if *FRT* and *loxP* sites are utilized, a chimeric FLP:Cre recombinase will be needed.

The open reading frames encoding the first and second recombinases may be directly fused to each other or may be joined by a linker that maintains the correct reading frame of the chimeric recombinase. It is understood that the recombinases may be fused amino to carboxy terminus, amino to amino terminus, or carboxy to amino terminus.

Genes encoding chimeric site-specific recombinases and recombination sites can be made using standard recombinant methods, synthetic techniques, or combinations thereof. Use of cloning vectors, expression vectors, adapters, and linkers is well known in the art and can be found in such references as Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed. (Cold Spring Harbor, New York, 1989). A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments and which choices can be readily made by those of skill in the art.

The FLP recombinase gene from yeast (*Saccharomyces cerevisiae)* is commercially available in plasmid pOG44 from Stratagene Cloning Systems (11011 North Torrey Pines Road, La Jolla, CA 92037). For a description of the FLP gene and various nucleic acids see, for example, Stratagene Cloning Systems, Catalogs 1995, 1996, 1997 (La Jolla, CA); and, Amersham Life Sciences, Inc, Catalog =97 (Arlington Heights, IL). Similarly, the sequences of many other site specific recombinases and their cognate recombination sites are publicly or commercially available. Genes encoding FLP and Cre can also be obtained, for example, by synthesizing the genes with mutually priming long oligonucleotides. See, for example, Ausubel *et al.* (eds.), Current Protocols In Molecular Biology, pages 8.2.8 to 8.2.13, Wiley Interscience (1990). Also, see Wosniak *et al.* (1987) *Gene* 60:115. Moreover, current techniques using the polymerase chain reaction provide the ability to synthesize genes as large as 1.8 kilobases in length (Adang *et al.* (1993) *Plant Mol. Biol.* 21:1131; Bombat *et al.* (1993) *PCR Methods and Applications* 2:266).

When the nucleic acid is prepared or altered synthetically, advantage can be taken of known codon preferences of the intended host where the nucleic acid is to be expressed. For example, although nucleic acid sequences of the present invention may be expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray *et al.* (1989) *Nucl. Acids Res.* 17:477-498; and Campbell *et al.* (1990) *Plant Physiol.* 92:1). Thus, the maize preferred codon for a particular amino acid may be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants are listed in Table 4 of Murray *et al.*, *supra*.

Examples of genes encoding recombinases, using maize preferred codons include, FLPm, described in co-pending application 08/972,258; the contents of which are incorporated herein by reference, and moCre, shown in SEQ. ID NOS. 1 and 2. FLPm is derived from the Saccharomyces 2μ plasmid FLP

recombinase, but is encoded by a nucleic acid sequence utilizing maize-preferred codons. While the FLPm nucleic acid sequence includes preferred codons for expression of amino acids in maize, it is understood that a useful sequence may contain codons occurring in maize with less than the highest reported maize codon frequencies. Examples of nucleic acids encoding chimeric recombinases include Cre:FLPm (SEQ. ID NO. 4), moCre:FLPm (SEQ. ID NO. 5), Cre:FLP (SEQ. ID NO. 6) and FLPm:Cre (SEQ. ID NO. 8).

The invention also provides expression cassettes containing a nucleic acid sequence encoding a chimeric site-specific recombinase, operably linked to a promoter that drives expression in a eukaryotic cell. Preferably the promoter is a plant promoter. For example, the plant expression vector PHP13147, shown in Figure 1, contains an expression cassette for Cre:FLPm, wherein the gene encoding the chimeric recombinase is operably linked to a ubiquitin promoter. As used herein "operably linked" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

As used herein "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells. Exemplary plant promoters include, but are not limited to, those that are obtained from plants, plant viruses, and bacteria genes that are expressed in plant cells such as those of *Agrobacterium* or *Rhizobium*. Both heterologous and non-heterologous (i.e., endogenous) promoters can be employed to direct expression of a sequence encoding a site-specific recombinase. The promoter may be constitutive, inducible or tissue specific.

Many different constitutive promoters can be utilized in the instant invention. Exemplary constitutive promoters include the promoters from plant viruses such as the 35S promoter from CaMV (Odell *et al.* (1985) *Nature* 313:810-812) and the promoters from such gene as rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); maize H3 histone (Lepetit *et al.* (1992) *Mol. Gen. Genet.* 231: 276-285 and Atanassova *et al.* (1992) *Plant Journal* 2(3):291-300); the 1'- or 2'-promoter derived from T-DNA of *Agrobacterium tumefaciens*, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (U.S. Patent No. 5,683,439), the *Nos* promoter, the Pemu promoter, the rubisco promoter, the GRP1-8 promoter, and other transcription initiation regions from various plant genes known to those of skill. The ALS promoter, a XbaI/NcoI fragment 5-prime to the *Brassica napus* ALS3 structural gene (or a nucleotide sequence that has substantial sequence similarity to said XbaI/NcoI fragment), represents a particularly useful constitutive promoter. (See co-pending Pioneer Hi-Bred International US Patent Application 08/409,297, the contents of which are incorporated by reference).

A variety of inducible promoters can be used in the instant invention. *See* Ward *et al.* (1993) *Plant Mol. Biol.* 22:361-366. Exemplary inducible promoters include that from the ACE1 system which responds to copper (Mett *et al.* (1993) *PNAS* 90:4567-4571); In2 gene from maize which responds to benzenesulfonamide herbicide safeners (Hershey *et al.* (1991) *Mol. Gen. Genetics* 227:229-237 and Gatz *et al.* (1994) *Mol. Gen. Genetics* 243:32-38); the Adh1 promoter which is inducible by hypoxia or cold stress, the Hsp70 promoter which is inducible by heat stress, and the PPDK promoter which is inducible by light; or Tet repressor from Tn10 (Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237. A particularly preferred inducible promoter is a promoter that responds to an inducing agent to which plants do not normally respond. An exemplary inducible promoter is

the inducible promoter from a steroid hormone gene the transcriptional activity of which is induced by a glucocorticosteroid hormone (Schena *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88:10421).

Examples of promoters under developmental control include promoters that initiate transcription only, or preferentially, in certain tissues, such as leaves, roots, fruit, seeds, or flowers. The operation of a promoter may also vary depending on its location in the genome. Thus, an inducible promoter may become fully or partially constitutive in certain locations.

The chimeric recombinase must be expressed in the plant cell in order for integration of the DNA of interest into the host chromosome. Accordingly, the expression cassette encoding the site-specific recombinase may be supplied *in cis* to the DNA of interest; *in trans* on a host chromosome or extrachromosomal replicon; or may be transferred to the host and transiently expressed near to the time that recombination is desired.

The compositions of the invention include transfer cassettes comprising nucleotide sequences encoding the chimeric recombinases of the invention. By transfer cassette is meant any nucleotide sequence that may be used to transform a cell of interest. For example, the transfer cassette may be an independent replicon such as a plasmid, shuttle vector, Ti plasmid, viral vector or the like. Alternatively, the transfer cassette could be a nucleic acid that is not capable of independent replication, yet could be transferred into an organism of interest by a variety of transformation protocols, such as particle bombardment, electroporation, and the like. Thus, the invention provides a transfer cassette comprising a nucleotide sequence encoding a recombinant protein comprising a first site-specific recombinase fused in frame with a second site-specific recombinase, wherein said nucleotide sequence is operably linked to a promoter that drives expression in a eukaryotic cell.

In the compositions and methods of the invention, the DNA of interest is flanked by target sites for two distinct site-specific recombinases. By

"flanked by" is meant that the recombination or target sites may be directly contiguous with the DNA of interest, or there may be one or more intervening sequences present between one or both ends of the DNA of interest and the site specific recombination sites. Intervening sequences of particular interest would include linkers, adapters, selectable markers and/or other sites which aid in vector construction or analysis and expression cassette for a gene of interest. Target sites for site-specific recombinases are known to those skilled in the art and are discussed in co-pending provisional application 60/065,613. Examples of target sites include, but are not limited to FRT, FRT1, FRT5, FRT6, FRT7, other FRT mutants, loxP, loxP mutants, and the like. See, for example, Schlake and Bode (1994) *Biochemistry* 33:12746-12751; Huang *et al.* (1991) *Nucleic Acids Research* 19:443-448; Sadowski (1995) In *Progress in Nucleic Acid Research and Molecular Biology* vol. 51, pp. 53-91; Cox (1989) *In Mobile DNA*, Berg and Howe (eds) American Society of Microbiology, Washington D.C., pp. 116-670; Dixon *et al.* (1995) 18:449-458; Umlauf and Cox (1988) *The EMBO Journal* 7:1845-1852; Buchholz *et al.* (1996) *Nucleic Acids Research* 24:3118-3119; Kilby *et al.* (1993) *Trends Genet.* 9:413-421: Rossant and Geagy (1995) *Nat. Med.* 1: 592-594; *Lox* Albert *et al.* (1995) *The Plant J.* 7:649-659: Bayley *et al.* (1992) *Plant Mol. Biol.* 18:353-361; Odell *et al.* (1990) *Mol. Gen. Genet.* 223:369-378; and Dale and Ow (1991) *Proc. Natl. Acad. Sci. USA* 88:10558-105620; Qui *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:1706-1710; Stuurman *et al.* (1996) *Plant Mol. Biol.* 32:901-913; and Dale *et al.* (1990) *Gene* 91:79-85; all of which are herein incorporated by reference.

By "target site for a site-specific recombinase" is meant a DNA sequence that is recognized by a particular site-specific recombinase. A variety of recombination sites are known to those skilled in the art and may be used in the methods and compositions of the invention. The site may have the sequence of the cognate site for a given recombinase, or may be modified, so long as it is capable of acting as a recombination site. The site may be contain the minimal sequences necessary for recombination, or it may contain additional sequences that enhance

recombination. Examples of recombination sites for use in the invention are known in the art and include FRT and *loxP* sites (See, for example, Schlake and Bode (1994) *Biochemistry* 33:12746-12751; Huang *et al.* (1991) *Nucleic Acids Research* 19:443-448; Paul D. Sadowski (1995) In Progress in Nucleic Acid Research and Molecular Biology vol. 51, pp. 53-91; Michael M. Cox (1989) *In Mobile DNA*, Berg and Howe (eds) American Society of Microbiology, Washington D.C., pp. 116-670; Dixon *et al.* (1995) 18:449-458; Umlauf and Cox (1988) *The EMBO Journal* 7:1845-1852; Buchholz *et al.* (1996) *Nucleic Acids Research* 24:3118-3119; Kilby *et al.* (1993) *Trends Genet.* 9:413-421: Rossant and Geagy (1995) *Nat. Med.* 1: 592-594; *Lox* Albert *et al.* (1995) *The Plant J.* 7:649-659: Bayley *et al.* (1992) *Plant Mol. Biol.* 18:353-361; Odell *et al.* (1990) *Mol. Gen. Genet.* 223:369-378; and Dale and Ow (1991) *Proc. Natl. Acad. Sci. USA* 88:10558-105620; Qui *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:1706-1710; Stuurman *et al.* (1996) *Plant Mol. Biol.* 32:901-913; Hartley *et al.* (1980) *Nature* 286: 860-864; Sauer (1994) *Current Opinion in Biotechnology* 5:521-527; and Dale *et al.* (1990) *Gene* 91:79-85; all of which are herein incorporated by reference.)

Each *loxP* and FRT site contains two 13 base pair inverted repeats which flank an 8 base pair spacer. The FRT site contains an additional non-essential 13 base pair repeat. The sequences of the *loxP* and FRT sites are shown in SEQ. ID NO. 1 and SEQ. ID NO. 2. A minimal FRT site comprising two 13 base pair repeats, separated by an 8 base spacer, is:

5'-GAAGTTCCTATTC[TCTAGAAA]GTATAGGAACTTC3'

wherein the nucleotides within the brackets indicate the spacer region. The nucleotides in the spacer region can be replaced with a combination of nucleotides, so long as the two 13-base repeats are separated by eight nucleotides. FLP is a conservative, site-specific recombinase, capable of catalyzing inversion of a nucleic acid sequence positioned between two inversely oriented FRTs; recombination between two molecules each containing a FRT site; and excision between FRT sites. The core region is not symmetrical, and its asymmetry dictates the directionality of

the reaction. Recombination between inverted FRT sites causes inversion of a DNA sequence between them, whereas recombination between directly oriented sites leads to excision of the DNA between them.

Nucleotide sequences containing a DNA of interest flanked by target sites, transfer cassettes for two distinct site-specific recombinases and vectors carrying these sequences may be constructed using standard molecular biology techniques. See, for example, Sambrook *et al.* (eds.) Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, cold Spring Harbor, NY 1989).

Techniques for transforming a wide variety of eukaryotic cells, including higher plant species are well known and described in the technical, scientific, and patent literature. See, for example, Weising *et al., Ann. Rev. Genet.* 22: 421-477 (1988). These methods are useful for transforming a plant cell with the chimeric recombinase expression cassettes of the invention and DNAs of interest flanked by target sites for the chimeric recombinase. The expression cassette encoding the site-specific recombinase may be present in the plant genome prior to transformation of the DNA of interest, or may be transformed into the plant around the time of transformation with the T-DNA to the plant cell so that it will be transiently expressed. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation, PEG poration, particle bombardment, silicon fiber delivery, or microinjection of plant cell protoplasts or embryogenic callus.

Agrobacterium tumefaciens-meditated transformation techniques are well described in the scientific literature. See, for example Horsch *et al., Science* 233: 496-498 (1984), Fraley *et al., Proc. Natl. Acad. Sci.* 80: 4803 (1983) and Kado, (1991), *Crit. Rev.Plant Sci.* 10:1. Descriptions of the *Agrobacterium* vector systems and methods for *Agrobacterium*-mediated gene transfer are provide in Gruber *et al., supra;* Miki, *et al., supra*; and Moloney *et al.* (1989), *Plant Cell Reports* 8:238. Although *Agrobacterium* is useful primarily in dicots, certain

monocots can be transformed by *Agrobacterium*. For instance, *Agrobacterium* transformation of maize is described in U.S. Patent No. 5,550,318. Other methods of agroinfection include *Agrobacterium rhizogenes*-mediated transformation (see, e.g., Lichtenstein and Fuller In: Genetic Engineering, vol. 6, PWJ Rigby, Ed., London, Academic Press, 1987; and Lichtenstein, C. P., and Draper, J,. In: DNA Cloning, Vol. II, D. M. Glover, Ed., Oxford, IRI Press, 1985),Application PCT/US87/02512 (WO 88/02405 published Apr. 7, 1988) describes the use of *A.rhizogenes* strain A4 and its Ri plasmid along with *A. tumefaciens* vectors pARC8 or pARC16.

Optimized methods and vectors for Agrobacterium-mediated transformation of plants in the family Graminae, such as rice and maize have been described by Heath *et al.* (1997) *Mol. Plant-Microbe Interact.* 10:221-227; Hiei *et al.* (1994) *Plant J.* 6:271-282 and Ishida *et al.* (1996) *Nat. Biotech.* 14:745-750, the contents of which are incorporated herein by reference. The efficiency of maize transformation is affected by a variety of factors including the types and stages of tissue infected, the concentration of *Agrobacterium*, the tissue culture media, the Ti vectors and the maize genotype. Super binary vectors carrying the vir genes of *Agrobacterium* strains A281 and A348 are useful for high efficiency transformation of monocots.

The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski *et al., Embo J.* 3: 2717-2722 (1984). Electroporation techniques are described in Fromm *et al., Proc. Natl. Acad. Sci.* 82: 5824 (1985). Ballistic transformation techniques are described in Klein *et al., Nature* 327: 70-73 (1987).

Viral means of introducing DNA into mammalian cells are known in the art. In particular, a number of vector systems are known for the introduction of foreign or native genes into mammalian cells. These include SV40 virus (See, e.g., Okayama *et al.* (1985) *Molec. Cell Biol. 5*:1136-1142); Bovine papilloma virus (See, e.g., DiMaio *et al.* (1982) *Proc. Natl. Acad. Sci. USA 79*:4030-4034);

adenovirus (See, e.g., Morin *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:4626; Yifan *et al.* (1995) *Proc. Natl. Acad. Sci. USA 92*:1401-1405; Yang *et al.* (1996) *Gene Ther. 3*:137-144; Tripathy *et al.* (1996) *Nat. Med. 2*:545-550; Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:2581-2584; Rosenfeld *et al.* (1991) *Science 252*:431-434; Wagner (1992) *Proc. Natl. Acad. Sci. USA 89*:6099-6103; Curiel *et al.* (1992) *Human Gene Therapy 3*:147-154; Curiel (1991) *Proc. Natl. Acad. Sci. USA 88*:8850-8854; LeGal LaSalle *et al.* (1993) *Science 259*:590-599); Kass-Eisler *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90*:11498-11502); adeno-associated virus (See, e.g., Muzyczka *et al.* (1994) *J. Clin. Invest. 94*:1351; Xiao *et al.* (1996) *J. Virol. 70*:8098-8108); herpes simplex virus (See, e.g., Geller *et al.* (1988) *Science 241*:1667; Huard *et al.* (1995) *Gene Therapy 2*:385-392; U.S. Patent No. 5,501,979); retrovirus-based vectors (See, for example, Curran *et al.* (1982) *J. Virol. 44*:674-682; Gazit *et al.* (1986) *J. Virol. 60*:19-28; Miller, A.D. (1992) *Curr. Top. Microbiol. Immunol. 158*:1-24; Cavanaugh *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:7071-7075; Smith *et al.* (1990) *Molecular and Cellular Biology 10*:3268-3271); herein incorporated by reference. See also, Wu *et al.* (1991) *J. Biol. Chem. 266*:14338-14342; Wu and Wu (*J. Biol Chem.* (1988)) *263*:14621-14624; Wu *et al.* (1989) *J. Biol. Chem. 264*:16985-16987; Zenke *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:3655-3659; Wagner *et al.* (1990) *87*:3410-3414.

DNA can also be introduced into plants by direct DNA transfer into pollen as described by Zhou *et al.*, *Methods in Enzymology*, 101:433 (1983); D. Hess, *Intern Rev. Cytol.*, 107:367 (1987); Luo *et al.*, *Plane Mol. Biol. Reporter*, 6:165 (1988). Expression of polypeptide coding genes can be obtained by injection of the DNA into reproductive organs of a plant as described by Pena *et al.*, *Nature*, 325.:274 (1987). DNA can also be injected directly into the cells of immature embryos and the rehydration of desiccated embryos as described by Neuhaus *et al.*, *Theor. Appl. Genet.*, 75:30 (1987); and Benbrook *et al.*, in *Proceedings Bio Expo* 1986, Butterworth, Stoneham. Mass., pp. 27-54 (1986). A variety of plant viruses

that can be employed as vectors are known in the art and include cauliflower mosaic virus (CaMV), geminivirus, brome mosaic virus, and tobacco mosaic virus.

Plants cells stably transformed with a chimeric recombinase expression cassette can be regenerated, e.g., from single cells, callus tissue or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues, and organs from almost any plant can be successfully cultured to regenerate an entire plant. Plant regeneration from cultured protoplasts is described in Evans *et al.*, *Protoplasts Isolation and Culture*, *Handbook of Plant Cell Culture*, Macmillilan Publishing Company, New York, pp. 124-176 (1983); and Binding, *Regeneration of Plants*, *Plant Protoplasts*, CRC Press, Boca Raton, pp. 21-73 (1985).

The regeneration of plants containing the recombinant genes can be achieved as described by Horsch *et al.*, *Science*, 227:1229-1231 (1985). In this procedure, transformants are grown in the presence of a selection agent and in a medium that induces the regeneration of shoots in the plant species being transformed as described by Fraley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 80:4803 (1983). This procedure typically produces shoots within two to four weeks and these transformant shoots are then transferred to an appropriate root-inducing medium containing the selective agent and an antibiotic to prevent bacterial growth. Transgenic plants of the present invention may be fertile or sterile.

Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee *et al.*, *Ann. Rev. of Plant Phys.* 38: 467-486 (1987). The regeneration of plants from either single plant protoplasts or various explants is well known in the art. See, for example, *Methods for Plant Molecular Biology*, A. Weissbach and H. Weissbach, eds., Academic Press, Inc., San Diego, Calif. (1988). This regeneration and growth process includes the steps of selection of transformant cells and shoots, rooting the transformant shoots and growth of the plantlets in soil. For maize cell culture and regeneration see generally, *The Maize Handbook*, Freeling and Walbot,

Eds., Springer, New York (1994); *Corn and Corn Improvement*, 3rd edition, Sprague and Dudley Eds., American Society of Agronomy, Madison, Wisconsin (1988).

One of skill will recognize that after a DNA, such as a chimeric recombinase expression cassette or target site for a chimeric recombinase is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

The methods and compositions of the invention are useful to integrate a DNA of interest into the genome of any host cell, including any plant host. As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. Plant cell, as used herein includes, without limitation, seeds suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants which can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. A particularly preferred monocot is maize. Other monocots of particular interest include wheat, rice, barley, sorghum and rye. Dicots of particular interest include soybean, Brassica, sunflower, alfalfa, and safflower.

Because of the use of the chimeric site-specifc recombinases and target sites provided herein, the cells transformed by the methods of the invention may be distinguishable from other transformation methods as the modified cells of the invention will contain nucleotide sequences of interest inserted into the genome flanked by target sites for distinct recombinases.

The following examples are offered by way of illustration not by way of limitation.

EXPERIMENTAL

Example 1

Construction of Vectors Containing a DNA of Interest Flanked By Target Sites For a Chimeric Site-Specific Recombinase

DNA fragments containing a DNA of interest flanked by *loxP* and FRT target sites are constructed either by synthesizing, annealing and ligating complementary oligonucleotides or by creating primers for PCR amplification of a DNA of interest with containing the loxP and FRT sites in addition to restriction sites useful for cloning into a vector of choice.

For example, long PCR primers may be designed wherein the 3' end of the primer hybridizes to the 5' end of the DNA of interest and the 5' end of the primers further contain *loxP or FRT* sites and useful cloning sites. The resulting PCR product is digested with the appropriate restriction enzyme and inserted into an appropriate vector.

Example 2

Excision of FRT Site by FLPm and the Cre:FLPm Chimeric Recombinase

A transfer cassette encoding a Cre-FLPm chimeric recombinase was transformed into plant cells having an expression cassette encoding GUS driven by the ubiquitin promoter, wherein a sequence flanked by either identical FRT or loxP sites interrupted the GUS open reading frame. Figures 2 and 3 show that the Cre-FLPm chimeric recombinase is functional independently at either the FRT site or the loxP site, as measured by the ability to activate GUS activity following excision of sequences between two identical target sites, thereby bringing GUS activity under the control of the ubiquitin promoter.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A recombinant protein comprising: a first site-specific recombinase fused in frame with a second site-specific recombinase.

2. The recombinant protein of claim 1, wherein said first and second site-specific recombinases are members of the integrase family of recombinases or active derivatives thereof.

3. The recombinant protein of claim 2, wherein said first and second recombinases are selected from the group consisting of Cre, FLP and active derivatives thereof.

4. A recombinant protein comprising the amino acid sequence of SEQ ID No. 3.

5. A recombinant protein comprising the amino acid sequence of SEQ ID No. 7.

6. A nucleotide sequence encoding a first site-specific recombinase fused in frame with a second heterologous site-specific recombinase.

7. The nucleotide sequence of claim 6, wherein said first and second site-specific recombinases are members of the integrase family of recombinases or active derivatives thereof.

8. The nucleotide sequence claim 7, wherein said first and second recombinases are selected from the group consisting of Cre, FLP and active derivatives thereof.

9. The nucleotide sequence of claim 8, wherein Cre and active derivatives thereof are encoded by *moCre* and derivatives thereof, and FLP and active derivatives thereof are encoded by *FLPm* and derivatives thereof.

10. A nucleotide sequence comprising SEQ ID No. 2.

11. A nucleotide sequence comprising SEQ ID No. 4.

12. A nucleotide sequence comprising SEQ ID No. 5.

13. A transfer cassette comprising a nucleotide sequence of claims 6-12, wherein said nucleotide sequence is operably linked to a promoter that drives expression in a eukaryotic cell.

14. A eukaryotic cell stably transformed with the transfer cassette of claim 13.

15. The eukaryotic cell of claim 14, wherein said cell is a plant cell.

16. A transformed plant having stably incorporated into its genome the transfer cassette of claim 13.

17. Seed of the plant of claim 16.

18. A method for integrating a DNA of interest into the genome of a eukaryotic cell, comprising:

a) transforming said cell with a transfer cassette comprising said DNA, wherein said DNA is flanked by a target site for a first site-specific recombinase and a target site for a second site-specific recombinase, and said genome contains at least one integration site comprising target sites corresponding to said target sites flanking said DNA; and

b) providing in said cell a recombinant protein comprising said first recombinase fused in frame with said second recombinase.

19. The method of claim 18, wherein said cell is a plant cell.

20. The method of claim 19, wherein said plant cell is monocotyledonous.

21. The method of claim 20, wherein said plant cell is maize, wheat, rice, barley, sorghum or rye.

22. The method of claim 19, wherein said plant cell is dicotyledonous.

23. The method of claim 22, wherein said plant cell is soybean, Brassica, sunflower, alfalfa or safflower.

24. The method of claim 18, wherein said first recombinase is Cre or an active derivative thereof and said second recombinase is FLP or an active derivative thereof.

25. A plant having stably integrated into a chromosome, at least one integration site comprising a target site for a first site-specific recombinase and a target site for a second site-specific recombinase, wherein, said target sites are contiguous or flank non-essential DNA.

26. The plant of claim 25, wherein said plant is a monocot.

27. The plant of claim 26, wherein said monocot is maize, wheat, rice, barley, sorghum or rye.

28. The plant of claim 25, wherein said plant is a dicot.

29. The plant of claim 28, wherein said plant is soybean, Brassica, sunflower, alfalfa or safflower.

30. Seed of the plant of claims 25-29.

31. A nucleotide sequence comprising a DNA of interest flanked by a target site for a first site-specific recombinase and a target site for a second site-specific recombinase.

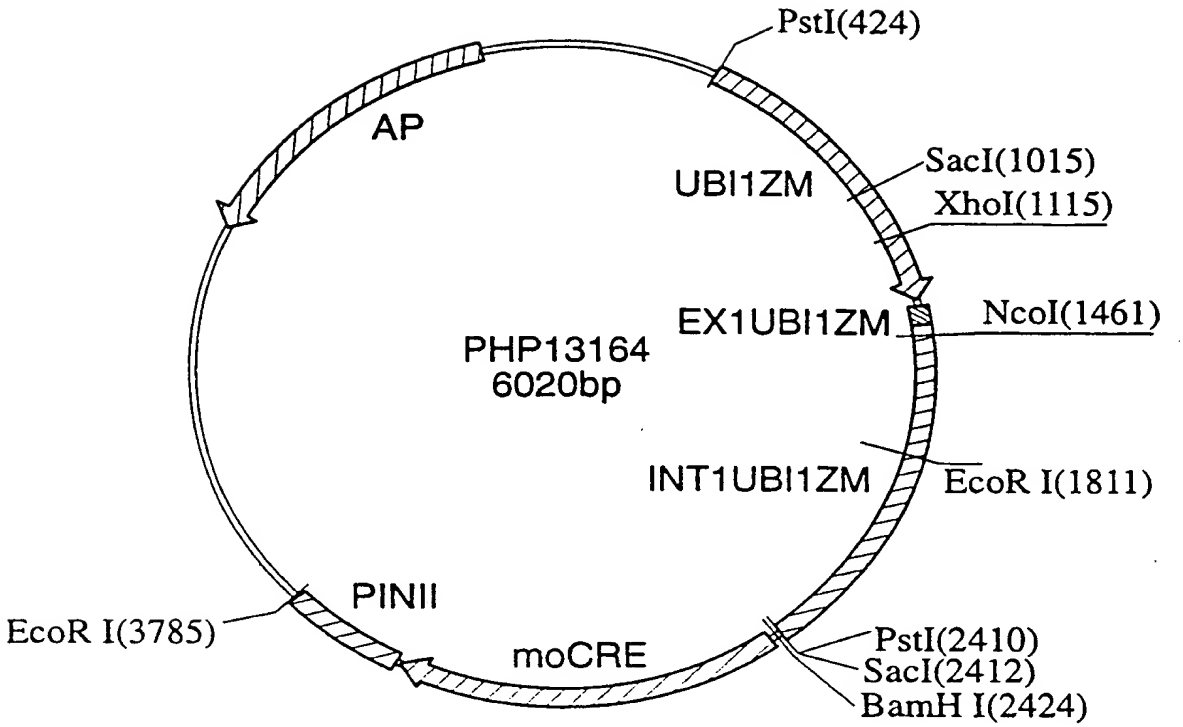
32. The nucleotide sequence of claim 31, wherein said recombinases are members of the integrase family of recombinases.

33. The nucleotide sequence of claim 32, wherein said recombinases are Cre and FLP.

34. A method for integrating a DNA of interest into the genome of a eukaryotic cell, comprising:

a) transforming said cell with a transfer cassette comprising said DNA, wherein said DNA is flanked by a target site for a first site-specific recombinase and a target site for a second site-specific recombinase, and said genome contains an integration site comprising target sites corresponding to said target sites flanking said DNA; and

b) providing in said cell said first recombinase and said second recombinase.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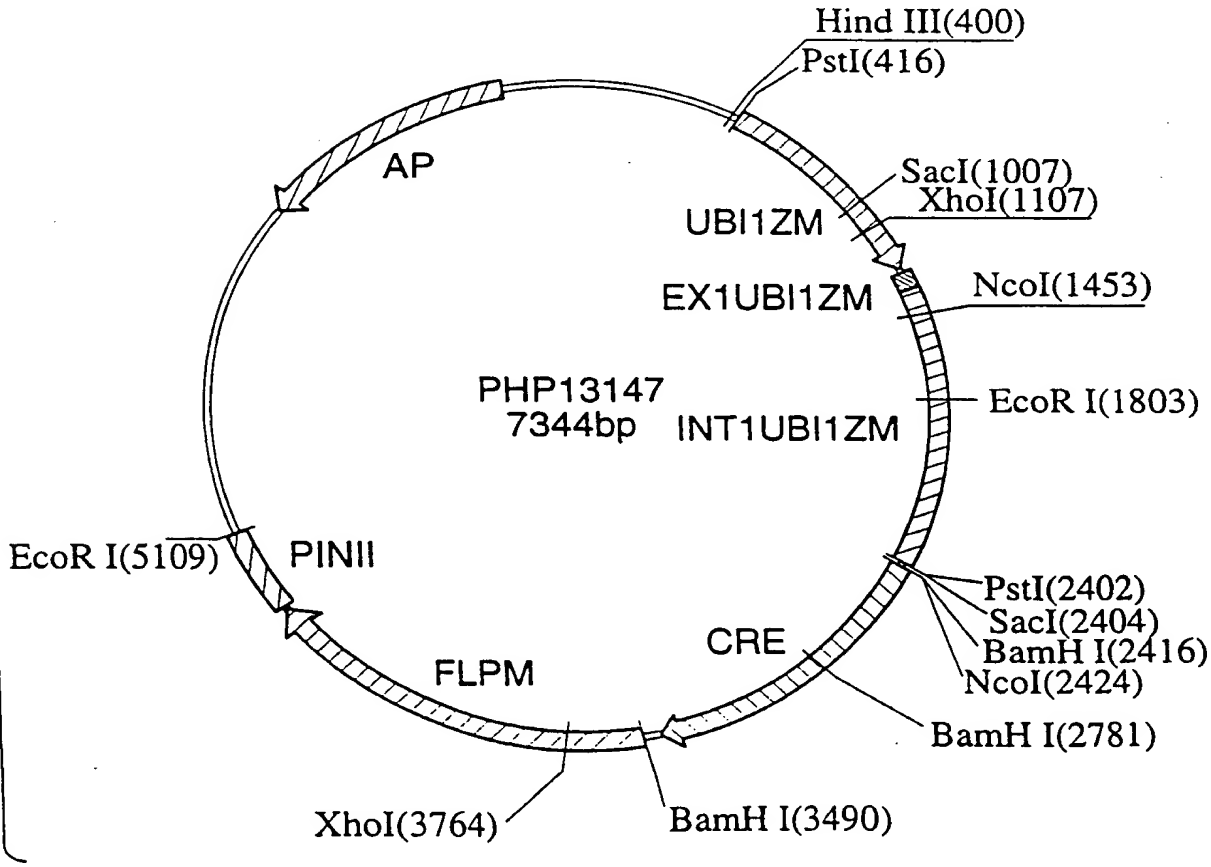
SacI(2412)
BamH I(2424)

FIG. 1.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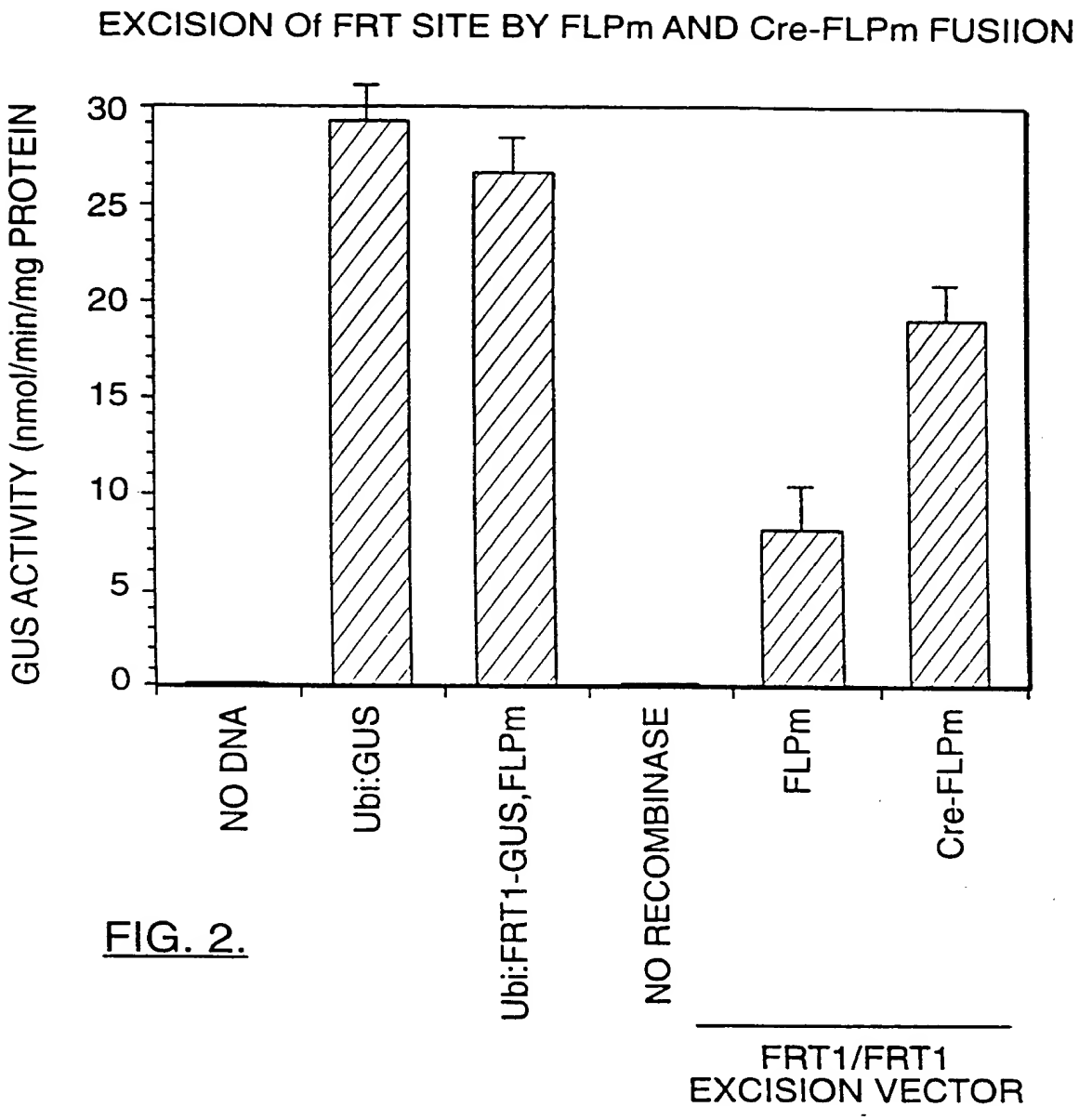
EXCISION Of FRT SITE BY FLPm AND Cre-FLPm FUSIION
GUS ACTIVITY (nmol/min/mg PROTEIN
30
25
20
15
10
5
0
NO DNA
Ubi:GUS
Ubi:FRT1-GUS,FLPm
NO RECOMBINASE
FLPm
Cre-FLPm
FRT1/FRT1
EXCISION VECTOR

FIG. 2.

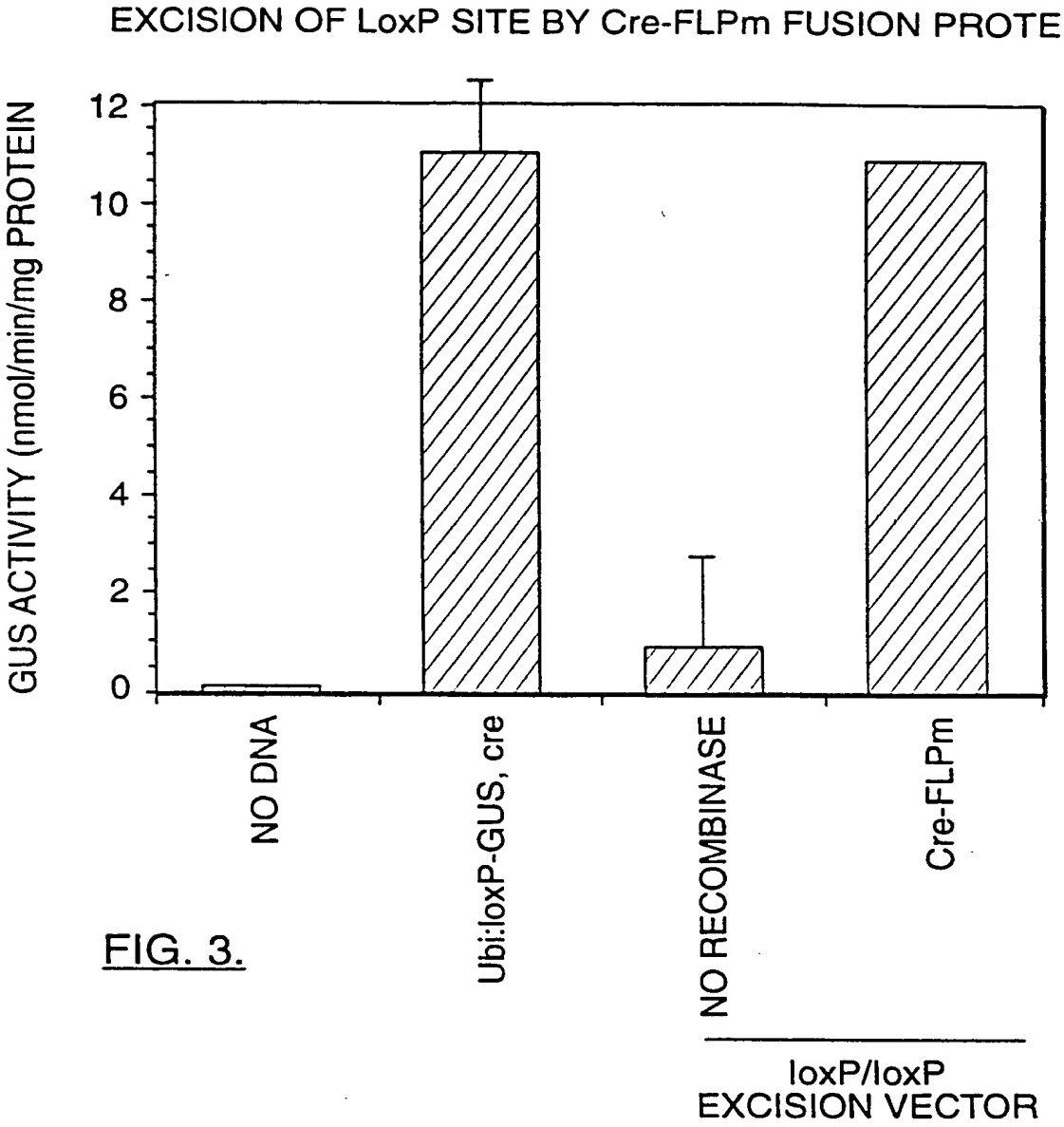
EXCISION OF LoxP SITE BY Cre-FLPm FUSION PROTEIN
GUS ACTIVITY (nmol/min/mg PROTEIN
0
2
4
6
8
10
12
NO DNA
Ubi:loxP-GUS, cre
NO RECOMBINASE
Cre-FLPm
loxP/loxP
EXCISION VECTOR

FIG. 3.

INTERNATIONAL SEARCH REPORT

Ir. ational Application No
PCT/US 98/24608

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N15/62 C12N15/82 C12N15/90 C12N5/10
A01H5/00 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 17176 A (PURDUE RESEARCH FOUNDATION) 4 August 1994 see esp. p.17 l.31 - p.20 l.30; figure 1A ---	31-34
A	WO 93 01283 A (US) 21 January 1993 see the whole document ---	1-34
A	SCHLAKE T ET AL: "USE OF MUTATED FLP RECOGNITION TARGET (FRT) SITES FOR THE EXCHANGE OF EXPRESSION CASSETTES AT DEFINED CHROMOSOMAL LOCI" BIOCHEMISTRY, vol. 33, no. 43, 1 November 1994, pages 12746-12751, XP000616165 cited in the application see esp. p.12751 l. par. --- -/--	1-34

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 1999	07/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 98/24608

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	ALBERT H. ET AL.: "Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome" THE PLANT JOURNAL, vol. 7, no. 4, 1995, pages 649-659, XP002097329 cited in the application see the whole document ---	1-34
A	SNAITH M R ET AL: "MULTIPLE CLONING SITES CARRYING LOXP AND FRT RECOGNITION SITES FOR THE CRE AND FLP SITE-SPECIFIC RECOMBINASES" GENE, vol. 166, no. 1, 1995, page 173/174 XP002061817 see the whole document ---	1-34
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